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Editor's Page

Welcome to the first issue of the Customs and Border Protection Laboratory Bulletin. During our hiatus many changes have occurred as our new title indicates. We have moved from the Department of Treasury to the Department of Homeland Security as part of the Bureau of Customs and Border Protection. Our move is not a change in direction but an expansion of our horizons.

This issue of the Customs and Border Protection Laboratory Bulletin focuses on articles that were in process at the beginning of our hiatus and during our accreditation process under ISO/IEC 17025. We thank the Savannah Laboratory for their contributions.

The Customs and Border Protection Laboratory Bulletin continues to solicit articles dealing with intriguing samples, innovative methods of analysis, and other topics of interest to Customs and related laboratories. Our primary focus is directed toward relevant applications to the type of analytical problems encountered by Customs chemists worldwide. You are invited to submit articles to this publication.

For further information on how to submit an article to the Customs and Border Protection Laboratory Bulletin, please contact the Editor at the following address:

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DETERMINATION OF BORON IN STEEL BY EMISSION SPECTROMETRY

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INTRODUCTION

Note 1(f) to Chapter 72 of the HTSUS defines “other alloy steel” by listing elements and quantities which when present result in a steel product being classified as “other alloy steel”. One of the elements listed is boron. The amount of boron required to render a product classifiable as other alloy steel is very low (0.0008% or 8ppm). Accurate quantification of boron in steel at such a low level has been a challenge for Customs laboratories. This paper investigates the use of optical emission spectrometry to quantify boron in steel at the 0.0008% level. The method used for the study was ASTM E 415-99a “Standard Test Method for Optical Emission Vacuum Spectrometric Analysis of Carbon and Low-Alloy Steel”. The instrument used in this study does not operate using a vacuum but rather uses an argon flush which provides an oxygen free atmosphere to transmit wavelengths below 200 nm. The applicable range for boron using ASTM E 415-99a is 0.0006% to 0.007%.

EXPERIMENTAL

Instrumentation: For the study a Spectrolab M Emission Spectrometer manufactured by SPECTRO Analytical Instruments was used. Operating parameters and calibration of the Spectrolab are preset by the manufacture: boron line -182.04 nm; internal reference standard line - iron 187.75 nm; counter electrode - tungsten with 3.4 mm analytical gap and auxiliary 5.6 mm gap. The instrument uses a 300 hz spark source with a 6 second exposure time.

Reference Materials: Certified Reference Materials from the National Institute of Standards and Technology (NIST), International Analysis Reference Material (IARM), National Bureau of Standards (NBS), and Brammer Standards Company (BS) were used in the study. Reference materials consisted of 32 mm diameter disk measuring 10 mm to 19 mm thick and rods measuring 3.2 mm in diameter by 51 mm long. Reference materials used are listed in Table 1.

Argon: The Spark Stand of the Spectrolab requires spectrometer quality argon (99.998% argon).

Procedure: The only sample preparation that was performed was to ensure that the surface of the Certified Reference Materials was clean and free of contamination. As recommended this was accomplished by abrading the materials using a Buehler 60 grit “zirconia” abrasive belt. NIST 665 and NIST 661 are 3.2 mm rods. These CRM were mounted onto a sample holder designed by Spectro for the analysis of wire rod samples. Each Certified Reference Material was analyzed eleven consecutive times. The first reading for each Certified Reference Material was discarded to eliminate possible “memory effect” from the previous reference material. The ten subsequent readings were recorded and a mean value established for each Certified Reference Material. The

mean value for each reference material was compared to the certified value for each reference material.

DISCUSSION AND RESULTS

The purpose of this study was to determine the suitability of emission spectrometry using a Spectrolab M Emission Spectrograph for determining the boron content in steel products at or near the 0.0008% (8 ppm) level. For the study only Certified Reference Materials were used. The Certified Reference Materials were either in the form of solid round discs or round wire rods. Round wire rods were included in the study because most of the samples received in the Savannah Laboratory for analysis are wire or wire rod. The boron concentration of the reference materials ranged from <0.0001% to 0.0026%. Six of the reference materials have boron content of less than 0.0008% and five of the reference materials have boron content of more than 0.0008%. A summary of the results of testing the Certified Reference Materials is shown in Table 1. The data obtained during the study are provided in Table 2.

This study determined that emission spectrometry using the Spectrolab M Emission Spectrometer is capable of determining the boron content in steel at very low levels; down to 0.0001%. With the exception of NIST #1261, analysis of the eleven Certified Reference Materials selected yielded values for boron that are within the published tolerance for the reference material. In the case of NIST #1261 the determined value was 0.0003% less than the certified value. The data show that for samples with a boron content near the 0.0008% tariff breakpoint the emission spectrograph provides a value for boron that is within the certified tolerances of the Certified Reference Materials. In every case had the Certified Reference Materials been samples submitted for tariff classification purposes the results of analysis using the Spectrolab M Emission Spectrometer would have resulted in the materials being correctly characterized for tariff purposes.

CONCLUSION

For HTS classification purposes it is necessary to determine the boron content of steel and steel products. Steels products having a boron content over 0.0008% are classified as alloy steel. Products having a boron content of 0.0008% or less are classified as nonalloy steel. This study determined that the emission spectrometry using the Spectrolab M Emission Spectrometer is capable of accurately determining the boron content in steel at levels ranging from 0.0001% to 0.0026%. The analysis is straightforward and requires minimal sample preparation.

Table 1 – Certified values for CRMs and Emission Spectrograph results

CRM	Certified Boron Content	Determined Boron Content
NBS# 1269*	<0.0001%	<0.0001%
NIST #665**	0.0001% \pm 0.0001	0.0001%
BS # 98*	0.0002% \pm 0.0001	0.0002%
NIST# 1261	0.0005% \pm 0.0001	0.0002%
NIST# 661**	0.0005% \pm 0.0001	0.0005%
IARM# 206A	0.0006% \pm 0.0002	0.0004%
NIST# 1263	0.0009% \pm 0.0001	0.0009%
NIST# 1263A	0.0009% \pm 0.0001	0.0009%
BS# 185A	0.0017% \pm 0.0002	0.0016%
NIST# 1262	0.0025% \pm 0.0001	0.0026%
BS# 86F	0.0026% \pm 0.0004	0.0029%

*Value for Boron is not certified

** Indicate the CRM is in the form of 51 mm X 3.2mm rods

Table 2 – Analytical data

Run #	NBS 1269	NIST 665	BS 98	NIST 1261	NIST 661	IARM 206A	NIST 1263	NIST 1263A	BS 185A	NIST 1262	BS 86F
1	<0.0001	0.0001	0.0002	0.0002	0.0005	0.0004	0.0008	0.0009	0.0016	0.0026	0.0028
2	<0.0001	0.0002	0.0002	0.0003	0.0004	0.0004	0.0009	0.0009	0.0016	0.0026	0.0027
3	<0.0001	0.0001	0.0002	0.0002	0.0006	0.0003	0.0009	0.0009	0.0016	0.0025	0.0027
4	<0.0001	0.0001	0.0002	0.0002	0.0005	0.0004	0.0010	0.0009	0.0017	0.0024	0.0030
5	<0.0001	0.0001	0.0001	0.0003	0.0007	0.0004	0.0009	0.0009	0.0016	0.0026	0.0028
6	<0.0001	0.0001	0.0002	0.0002	0.0005	0.0004	0.0009	0.0009	0.0016	0.0026	0.0028
7	<0.0001	0.0001	0.0002	0.0002	0.0006	0.0004	0.0009	0.0008	0.0016	0.0026	0.0030
8	<0.0001	0.0001	0.0002	0.0002	0.0004	0.0004	0.0009	0.0008	0.0016	0.0027	0.0032
9	<0.0001	0.0001	0.0001	0.0002	0.0005	0.0004	0.0009	0.0009	0.0016	0.0025	0.0031
10	<0.0001	0.0001	0.0002	0.0002	0.0004	0.0004	0.0009	0.0009	0.0016	0.0025	0.0030
Mean	<0.0001	0.0001	0.0002	0.0002	0.0005	0.0004	0.0009	0.0009	0.0016	0.0026	0.0029
S	0.0000	0.0000	0.00003	0.00003	0.0001	0.00003	0.00004	0.00004	0.00003	0.0001	0.0002

COMPARISON OF IMAGE ANALYSIS VERSUS MICROPROJECTOR METHOD FOR DETERMINING WOOL FINENESS

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INTRODUCTION

Wool imported into the United States is classified in the HTSUS according to the fineness or grade of the fibers. Wool grade is a numerical designation used in classifying wool in its raw, semi-processed, and processed forms based on the average fiber diameter and variation of fiber diameter of the wool. The diameter of fibers is measured in microns (micrometers).

The recognized method for determining wool grade is ASTM D 2130 (adopted as USCL 51-06). ASTM D 2130 requires the use of an obsolete microprojector that is no longer manufactured and for which service and parts are no longer available. For this reason a study was initiated to determine the feasibility of using a modern image analysis system for determining the fineness of imported wool. At the time of this study 75 samples of wool were analyzed by microprojector according to ASTM D 2130 and by state-of-the-art image analysis. Thirty-one of the samples were known reference standards and forty-four of the samples were from imported wool. The results were compared to determine if image analysis is a suitable alternative to microprojection as a means of determining the grade of wool.

EQUIPMENT AND MATERIALS

Microprojector- Baush & Lomb microprojector equipped with a fixed body tube, a focusable stage, focusable substage with a condenser and iris diaphragm, and vertically installed light source to give a precise magnification of 500X.

Image Analysis System – Olympus BH-2 polarizing light microscope in non-polarizing mode with Bioquant Meg IV image analysis system.

Heavy Duty Sectioning Device

Microscope Slides and Cover Glasses

Mounting Medium – Colorless immersion oil with a refractive index of 1.480 ± 0.005 at 68 degrees F (20 degrees C), and a viscosity of 78.8 SUS at 100 degrees F (37.8 degrees C).

PROCEDURE

All test specimens were preconditioned and taken from pre-conditioned clean yield samples or standard wool samples supplied by U.S. Testing Co. Specimen slides were prepared in accordance with procedures of ASTM D 2130. Analysis by microprojector

was conducted according to ASTM D 2130 using a wedge card to record individual fiber measurements. Analysis by image analysis was accomplished by marking fiber borders near the mid-length of a fiber and having the system calculate fiber diameter and record individual measurements. In cases where less than 1000 fibers were required to be measured and when time permitted the same specimen slide was measured by both microprojector and image analysis.

It should be noted that ASTM D 2130 requires that only fibers whose mid-length area falls within the field of the 4 inch (100mm) diameter circle, centrally located in the projected area were measured whereas using image analysis all fibers in the field of view were measured.

RESULTS AND DISCUSSION

Specimens analyzed spanned the full range of wool grades from 80's to less than 36's. For each of the 31 standard wool samples the grade determined by microprojector and by image analysis was the same. In each case the correct grade was assigned. The results of analyses are shown in table 1. Forty-four samples of imported wool were analyzed as part of this study. For thirty-nine of the samples analysis by microprojector and image analysis resulted in the same grade being assigned to the sample. For three of the samples analysis by microprojector and image analysis resulted in different grades being assigned. In each case the grade assignments were different by one grade (e.g. analysis by microprojection resulted in a grade assignment of 60's and analysis by image analysis resulted in a grade assignment of 62's). In each case the grade assignment resulting from analysis by image analysis was one grade higher than that for analysis by microprojector. For a screening method this was viewed as acceptable. The reason for the difference in the three samples may be attributed to the practice using image analysis of measuring all fibers in the field of view. Using the microprojector only fibers falling near the center of the field of view are measured. The reason for this is image distortion of fibers far from the center which may result in inaccurate measurements. The results of analyses are shown in table 2.

CONCLUSION

Of the 75 samples analyzed for this study analysis by image analysis resulted in the same grade assignment as analysis by microprojector ninety-six percent of the time with a different grade assignment in three cases. When examining standard wool samples the grade assignment was the same in every case. The study indicates that use of image analysis for determining wool grade is suitable as a screening technique. Because the technique did not produce the same result as the microprojector in every case, in instances where analysis by image analysis results in assignment of a grade which is different than that claimed by the importer the sample should be analyzed by microprojector in accordance with ASTM D 2130.

REFERENCES

1. Standard test Method for Diameter of Wool and other Animal Fibers by Microprojection, ASTM D 2130.

2. U.S. Testing Company for the Measurement Schedule for Designating grades of Wool.

Table 1 – Samples

MP: Microprojector IA: Image Analysis System

Method	Country of Origin	Number of Fibers Measured	Average Diameter	Standard Deviation	Grade ('s)
MP	Australia	785	21.03	4.49	64
IA		600	22.00	5.02	64
MP	Australia	901	23.28	4.74	62
IA		808	22.38	5.31	62
MP	Australia	903	20.61	4.38	64
IA		627	21.24	5.04	64
MP	Australia	1193	21.12	4.80	64
IA		814	20.93	4.96	64
MP	Australia	933	21.31	5.58	62
IA		706	21.60	5.24	62
MP	Australia	1094	22.01	4.24	64
IA		611	21.08	4.55	64
MP	Australia	944	22.95	3.70	62
IA		866	21.30	5.43	62
MP	Australia	986	23.76	4.50	60
IA		833	23.71	6.10	60
MP	Uruguay	1152	24.39	6.61	58
IA		1846	23.25	6.74	58
MP	Australia	1830	32.15	7.89	48
IA		1802	31.29	8.52	48
MP	New Zealand	2593	36.03	8.03	44
IA		2414	35.86	10.08	44
MP	Australia	1424	25.65	5.43	58
IA		1210	24.79	6.45	60
MP	Australia	1361	23.71	4.54	60
IA		815	22.51	5.21	62
MP	Australia	1934	20.23	4.61	64
IA		255	19.93	4.87	64

MP	New Zealand	1232	27.63	6.32	56
IA		1218	26.49	7.19	56
MP	Australia	1570	20.80	5.27	62
IA		1012	20.77	5.41	62
MP	Australia	747	21.52	3.63	64
IA		901	19.25	4.73	64
MP	Australia	660	20.78	3.77	64
IA		608	21.24	5.10	64
MP	Australia	651	21.69	3.88	64
IA		909	21.74	4.60	64
MP	Australia	815	23.85	4.70	60
IA		807	24.06	4.69	60
MP	Australia	809	20.76	4.17	64
IA		603	20.80	4.50	64
MP	Australia	1304	25.55	5.52	58
IA		846	24.34	6.39	60
MP	Australia	785	21.03	4.49	64
IA		600	22.00	5.02	64
MP	New Zealand	1982	37.60	9.05	40
IA		920	36.60	7.99	40

MP	Australia	833	20.32	4.91	64
IA		1134	21.32	5.02	64
MP	Australia	915	23.00	4.54	62
IA		833	21.61	5.41	62
MP	Australia	1188	20.82	4.77	64
IA		621	19.57	4.94	64
MP	New Zealand	2815	38.28	5.07	36
IA		2420	36.84	10.02	36
MP	Australia	1224	21.05	4.88	64
IA		623	20.26	4.89	64
MP	Australia	689	23.47	4.39	62
IA		839	22.05	5.09	62
MP	Australia	948	20.53	4.28	64

IA		710	19.42	5.13	64
MP	New Zealand	2658	40.90	5.70	>36
IA		2200	40.92	9.57	>36
MP	Australia	1134	21.81	4.22	64
IA		833	21.91	4.92	64
MP	Australia	1204	21.32	5.02	64
IA		817	20.32	4.91	64
MP	New Zealand	1149	25.70	5.76	58
IA		1030	26.08	6.24	58
MP	Australia	1830	32.15	7.89	48
IA		1802	31.29	8.52	48

MP	New Zealand	2593	36.03	8.03	44
IA		2414	35.86	10.08	44
MP	Australia	911	22.48	4.57	62
IA		831	20.66	5.24	62
MP	New Zealand	2557	40.46	10.91	>36
IA		1301	43.89	16.50	>36
MP	Australia	920	36.60	7.99	40
IA		1984	37.60	9.05	40
MP	Australia	1163	24.67	5.93	58
IA		1106	25.02	6.14	58
MP	Australia	1428	25.45	5.34	58
IA		1213	25.13	5.47	58
MP	New Zealand	2442	37.34	9.67	40
IA		2415	36.52	9.43	40
MP	Australia	1103	25.42	5.92	58
IA		1009	26.07	6.49	58

Table 2 - Standards

MP: Microprojector IA: Image Analysis System

Method	Country of Origin	Number of Fibers Measured	Average Diameter	Standard Deviation	Grade ('s)
MP	Standard	379	28.55	4.80	54
IA		407	29.42	6.52	54
MP	Standard	888	32.98	9.28	46
IA		546	31.50	9.99	46
MP	Standard	158	39.65	10.05	36
IA		309	38.95	9.25	36
MP	Standard	2026	33.18	9.54	46
IA		1150	33.78	9.76	46
MP	Standard	1828	31.33	8.27	48
IA		1854	32.12	8.34	48
MP	Standard	2420	36.30	10.37	40
IA		2450	36.64	10.60	40
MP	Standard	1252	28.57	7.53	56
IA		1219	27.69	7.12	56
MP	Standard	721	21.19	5.01	64
IA		815	21.86	4.85	64
MP	Standard	2316	36.19	8.90	44
IA		2227	35.87	8.20	44
MP	Standard	576	18.14	3.62	80
IA		600	17.96	3.40	80
MP	Standard	928	23.40	5.78	62
IA		947	23.05	5.42	62

MP	Standard	420	20.33	4.56	70
IA		436	19.14	4.29	70
MP	Standard	927	23.65	6.37	60
IA		919	24.91	6.19	60
MP	Standard	2419	37.14	9.98	40
IA		2452	38.06	10.52	40

MP	Standard	2667	39.99	10.21	36
IA		2699	38.97	10.47	36
MP	Standard	1447	28.29	8.01	54
IA		1008	29.11	8.20	54
MP	Standard	1049	25.12	5.82	58
IA		1265	25.46	6.15	58
MP	Standard	1623	30.29	7.45	50
IA		1553	30.31	6.48	50

Table 3 – Standard Wool Tops

MP: Microprojector IA: Image Analysis System

Method	Country of Origin	Number of Fibers Measured	Average Diameter	Standard Deviation	Grade ('s)
MP	Standard	409	17.96	2.98	80
IA		523	18.00	3.54	80
MP	Standard	438	19.42	3.87	70
IA		525	19.58	4.22	70
MP	Standard	400	20.97	4.68	64
IA		439	21.15	4.79	64
MP	Standard	673	22.27	5.27	62
IA		739	22.59	5.39	62
MP	Standard	893	23.97	5.44	60
IA		975	24.08	5.59	60
MP	Standard	1050	24.68	6.87	58
IA		1100	25.87	8.03	58
MP	Standard	1209	27.13	6.01	56
IA		1005	27.73	6.47	56
MP	Standard	1876	28.12	7.98	54
IA		1905	28.60	8.11	54
MP	Standard	1997	29.97	7.19	50
IA		523	30.52	7.57	50
MP	Standard	2103	32.18	7.90	48
IA		1123	32.45	7.40	48
MP	Standard	2279	33.87	9.37	46
IA		1164	34.25	9.56	46
MP	Standard	476	36.76	8.23	40
IA		527	37.49	8.37	40
MP	Standard	695	38.90	9.51	36
IA		473	39.76	9.37	36

THE DETERMINATION OF THE COUNTRY OF ORIGIN OF ORANGE JUICE AND APPLE JUICE USING TRACE METAL PROFILES AS DETERMINED BY HIGH RESOLUTION ICP/MS AND MULTIVARIATE STATISTICS

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INTRODUCTION

It has been reported that the concentration of trace elements in juice is directly related to the abundance of these components in the soil. Scientists¹ have reported geographic characterization of orange juice from Florida, Israel, and Brazil using trace metals combined with stable isotopic deuterium/hydrogen ratios. A considerable effort in defining geographic origin of orange juice using trace metals has also been done by Nikdel et. al² from the Florida Department of Citrus.

Customs and Border Protection (CBP) has an interest in determining the country of origin of various agricultural products in order to enforce existing importation laws and regulations and to collect the appropriate tariffs as provided by law. As do other countries, the United States allows some agricultural imports from small underdeveloped countries without tariff assessment, however, there may be limits imposed on such imports. In many instances, countries which are required to pay a tariff in order to import their products to the U.S. will attempt to circumvent the tariff by transshipping the product through one of the "tariff free" countries.

CBP Laboratories have been working on laboratory methods to facilitate scientific identification of the country of origin of various agricultural products. One such method presented here includes the determination of the trace metal profile of the product for comparison with an established database of trace metal profiles from various countries.

The method while simplistic in nature does require careful control over several of the experimental parameters. Obviously the country of origin prediction is only as good as the database that is being used for comparison. The data in the database should be representative of the countries in question and should include samples taken throughout the growing season of the country. Periodic updates of sampling may be required in order to assess possible seasonal variations. Other requirements include careful attention to analytical protocols and quality assurance considerations in order to produce verifiable and defensible data. Also, experience with multivariate statistics is essential in order to successfully build and utilize a suitable database for making country of origin predictions.

EXPERIMENTAL

Juice samples are normally concentrated prior to shipment to the U.S. in order to lower shipping costs. The degree of concentration is termed "brix" which is a measure of

sugars in the juice and can be measured by refractometer. Typical apple and orange juice concentrates have brix values of around 70 and 65 respectively while single strength juices have brix values of 13.3 and 11.8 respectively. All trace metal concentrations are normalized to single strength juice prior to making country-of-origin predictions.

Preparation of the juice samples for trace metal analyses requires acid digestion in order to solubilize the sample. Typically a 0.5 gm sample of the juice concentrate is weighed directly into a Teflon digestion vessel and 10 ml of EM Merck Omni-Trace HNO_3 is added. A set of ten samples may be digested at the same time using a CEM 2100 microwave digestion system. The specific microwave settings are shown in Table 1. After digestion, the digestate is diluted to 50 ml with 18 M Ω cm water from a Milli-Q Water system (Elix10 + Gradient A10), manufactured by Millipore Corp. Additional dilutions of the sample are made as necessary with the addition of 1 ppb of indium as an internal standard. The internal standard is necessary to correct for instrumental drift, which is inherent with ion multipliers, and the procedure is common in ICP/MS analyses.

A Finnigan Element-2 high resolution ICP/MS was used in all trace metal analyses reported here. Isobaric interferences common in ICP/MS analyses are virtually eliminated by selecting the resolution power needed to resolve the isobaric species from the desired element. The instrument has a resolution power of approximately 300, 4000, and 10000 at low medium and high-resolution modes respectively. The instrument requires daily tuning and mass calibration in each of the resolution modes. Typical detector count rates for a 1-ppb indium standard are 1.6×10^6 CPS, 120,000 CPS and 10,000 CPS in low, medium and high resolution modes. Obviously then, higher resolving power comes with a substantial sacrifice in sensitivity. Fortunately, most elements can be successfully analyzed in either low or medium resolution modes. A CETAC ASX-100 auto-sampler is used for sample introduction into the ICP/MS. The auto-sampler has a built in Plexiglas cover, which protects the uncovered samples from airborne contaminants present in the instrument room.

Instrument standardization performed in this work includes a five-point calibration curve with a correlation coefficient of 0.995 or better. The calibration curve for each element is verified prior to sample analyses by analyzing an initial calibration verification standard (ICV) which is a standard from a source other than the calibration standards. A continuing calibration standard (CCV) is analyzed after each ten samples throughout the sample run in order to verify that the instrument calibration is maintained. The criterion for acceptance for the ICV and CCV is $\pm 10\%$. A certified reference sample from NIST (SRM1570A Spinach leaf) is included with each sample run to validate the data. Also at least one reagent blank is included with each sample run. The Finnigan software will create a Text file of the raw data that can be imported into an Excel spreadsheet for making final concentrations in ppm by correcting for dilutions, sample mass, blank contributions, and correction for degree of concentration (brix).

Table I
Microwave Digestion Program
for
Agricultural Products

Stage	(1)	(2)	(3)	(4)	(5)
Power	50%	70%	70%	70%	70%
Pressure (psi)	100	200	300	350	375
Run Time (min)	7:00	4:00	4:00	4:00	4:00
Time @ Press	4:00	4:00	4:00	4:00	4:00
Temperature	130°C	160°C	160°C	160°C	160°C

Number of Vessels 10

Fourteen elements were analyzed for inclusion into the reference database for country of origin prediction. These elements include barium, boron, calcium, copper, phosphorous, iron, magnesium, potassium, manganese, rubidium, sodium, silicon, strontium, and zinc. A text file of the element concentrations along with the country of origin identifications is imported into the multivariate statistics software (SAS Institute version 8.1). A Stepwise Discriminate Analysis is performed to select those elements having sufficient discriminatory power to distinguish one country's metal data from another. The stepwise discriminant analysis is based upon the significance level of the F-Test. Tables 2 and 3 show the elements selected by SAS and their concentrations. Once a prediction model has been established using the elements selected above, the model should be tested for its accuracy by doing discriminant analysis using resubstitution and/or cross validation. Resubstitution creates a calibration data set and a prediction data set and places all of the reference samples in each data set. The software then produces a prediction accuracy of the model using this criterion. Cross validation is a bit more rigorous method that removes one reference sample at a time from the calibration data set and places this reference sample as an unknown sample in the prediction data set for accuracy prediction. This process is repeated until all reference samples have been placed in the prediction set.

Once the prediction accuracy of the model is acceptable, the user is ready to begin making country of origin predictions on unknown samples. A text file of the trace metal concentrations of the samples of unknown origin are appended to the reference data file with the country of origin field left blank on the unknown samples. A discriminant analysis may now be performed using only the elements selected from the stepwise discriminant analysis above. The software will predict the country of origin of the samples and report the probability match with the reference samples from that country. It should be noted that this prediction is based upon the proximity of the sample concentrations to the centroid values calculated in the reference database.

A visual representation of the data may be produced by plotting the canonical variables produced from a canonical discriminant analysis. This allows the user to visualize the proximity of the samples of unknown origin with the reference countries. Canonical discriminant analysis produces one fewer canonical variables than either the number of countries or the number of elements in the data set, whichever is least. Canonical variables are weighted linear combinations of the trace metal variables with the first canonical variable having the most discriminatory power and each succeeding variable sequentially less discriminatory power. The canonical variable data may be output to a file for plotting within SAS or may be exported to other plotting software. Figures 1, 2, and 3 demonstrate the utility of plotting these variables using SigmaPlot.

Table 2
Orange Juice Trace Metal Data

Sample	Country	B ppm	Na ppm	Si ppm	Fe ppm	Sr ppm	Ba ppm
SV010602A	Belize	0.69	3.71	0.54	0.74	0.16	0.20
SV010602B	Belize	0.72	2.95	0.34	0.68	0.15	0.20
SV010603A	Belize	0.61	2.82	0.32	0.69	0.17	0.21
SV010603B	Belize	0.61	2.95	0.25	0.69	0.17	0.21
SV010604A	Belize	0.60	3.76	0.39	0.63	0.22	0.24
SV010604B	Belize	0.59	3.44	0.37	0.67	0.22	0.23
Mean		0.64	3.27	0.37	0.68	0.18	0.22
Variance		0.003	0.174	0.010	0.001	0.001	0.000
SV010563-162A	Brazil	0.72	3.22	0.79	0.65	0.40	0.32
SV010563-162B	Brazil	0.70	2.71	0.83	0.63	0.39	0.32
SV010913A	Brazil	0.62	2.29	1.00	0.60	0.32	0.25
SV010913B	Brazil	0.65	2.11	0.73	0.54	0.36	0.23
SV010459-1	Brazil	0.45	1.77	1.23	0.53	0.29	0.22
SV010459-2	Brazil	0.46	1.91	1.09	0.53	0.27	0.22
SV010575A	Brazil	0.68	4.34	2.33	0.42	0.26	0.18
SV010575B	Brazil	0.71	4.26	2.59	0.42	0.26	0.19
Mean		0.63	2.83	1.32	0.54	0.32	0.24
Variance		0.012	1.041	0.524	0.007	0.003	0.003
SV010599A	Costa Rica	0.89	2.59	0.44	0.56	0.41	0.40
SV010599B	Costa Rica	0.89	2.04	0.39	0.56	0.41	0.42
SV010600A	Costa Rica	0.87	0.93	0.20	0.48	0.38	0.35
SV010600B	Costa Rica	0.83	1.15	0.34	0.50	0.38	0.35
SV010601A	Costa Rica	0.79	7.79	0.56	0.51	0.47	0.47
SV010601B	Costa Rica	0.86	7.73	0.44	0.47	0.47	0.45
Mean		0.86	3.70	0.40	0.51	0.42	0.40
Variance		0.002	10.21	0.015	0.001	0.001	0.003

Table 3
Apple Juice Trace Metal Data

Country	Statistics	B ppm	Si ppm	P ppm	Fe ppm	Rb ppm	Sr ppm	Ba ppm
US	Mean	3.05	2.67	81.2	0.50	0.53	0.17	0.22
	Std Dev	0.81	2.33	8.99	0.28	0.19	0.08	0.08
	Variance	0.65	5.44	80.7	0.078	0.035	0.007	0.007
Austria	Mean	1.65	1.74	57.1	0.75	0.94	0.05	0.05
	Std Dev	0.34	2.18	3.09	0.26	0.53	0.05	0.02
	Variance	0.11	4.74	9.54	0.07	0.28	0.002	0.001
New Zealand	Mean	2.09	4.62	56.2	0.37	0.73	0.14	0.07
	Std Dev	0.22	1.75	4.74	0.20	0.15	0.02	0.02
	Variance	0.05	3.07	22.5	0.042	0.024	0.000	0.000
Hungary	Mean	1.42	1.28	58.3	1.3	0.60	0.06	0.06
	Std Dev	0.35	1.62	10.0	0.37	0.07	0.05	0.06
	Variance	0.12	2.61	100	0.14	0.006	0.003	0.003
Brazil	Mean	2.45	5.18	52.9	0.25	1.36	0.09	0.06
	Std Dev	0.19	1.26	4.33	0.09	0.07	0.01	0.01
	Variance	0.04	1.59	18.8	0.01	0.005	0.000	0.000
Italy	Mean	2.70	1.41	58.6	0.61	0.91	0.14	0.07
	Std Dev	0.15	0.55	2.26	0.27	0.11	0.02	0.02
	Variance	0.02	0.30	5.13	0.073	0.012	0.000	0.000
Chile	Mean	3.41	3.88	72.1	0.51	2.68	0.23	0.13
	Std Dev	0.38	1.57	11.2	0.45	0.45	0.02	0.03
	Variance	0.14	2.46	124.8	0.21	0.21	0.001	0.001

RESULTS AND DISCUSSION

Figure 1 clearly demonstrates the capability of discerning the geographic origin of orange juice between Brazil, Costa Rica, and Belize. The five unknown samples shown in figure 1 are real samples, one, which clearly belongs to Costa Rica, and one, which clearly belongs to Brazil. The other three unknown samples appear to belong to a part of Brazil not covered by our database or possibly a third country not in our database. Another possibility is that these three samples are blends of Costa Rican and Brazilian juices.

Canonical plots of apple juice reference samples from seven countries are shown in figure 2. Generally the geographic origins of the samples are distinguishable, however some overlapping does occur. Particularly bothersome are the samples from the U.S. and New Zealand. However, by plotting canonical variable 1 versus variable 3 as shown in figure 3, samples from the U.S. are easily separated from those of New Zealand.

Therefore a combination of plots may be necessary in order to separate overlapping samples.

CONCLUSIONS

This work demonstrates the feasibility of determining the geographic origin of agricultural products using trace metal profiles. Establishment of a representative reference database is essential for this work. Tables 2 and 3 demonstrate that the trace metal concentrations vary from country to country on the order of tens to hundreds of parts-per-billion. This requires very careful analytical measurements with careful attention to reagent blanks and the use of standard reference materials.

ACKNOWLEDGEMENTS

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Figure 1
Canonical Plot of Orange Juice Samples

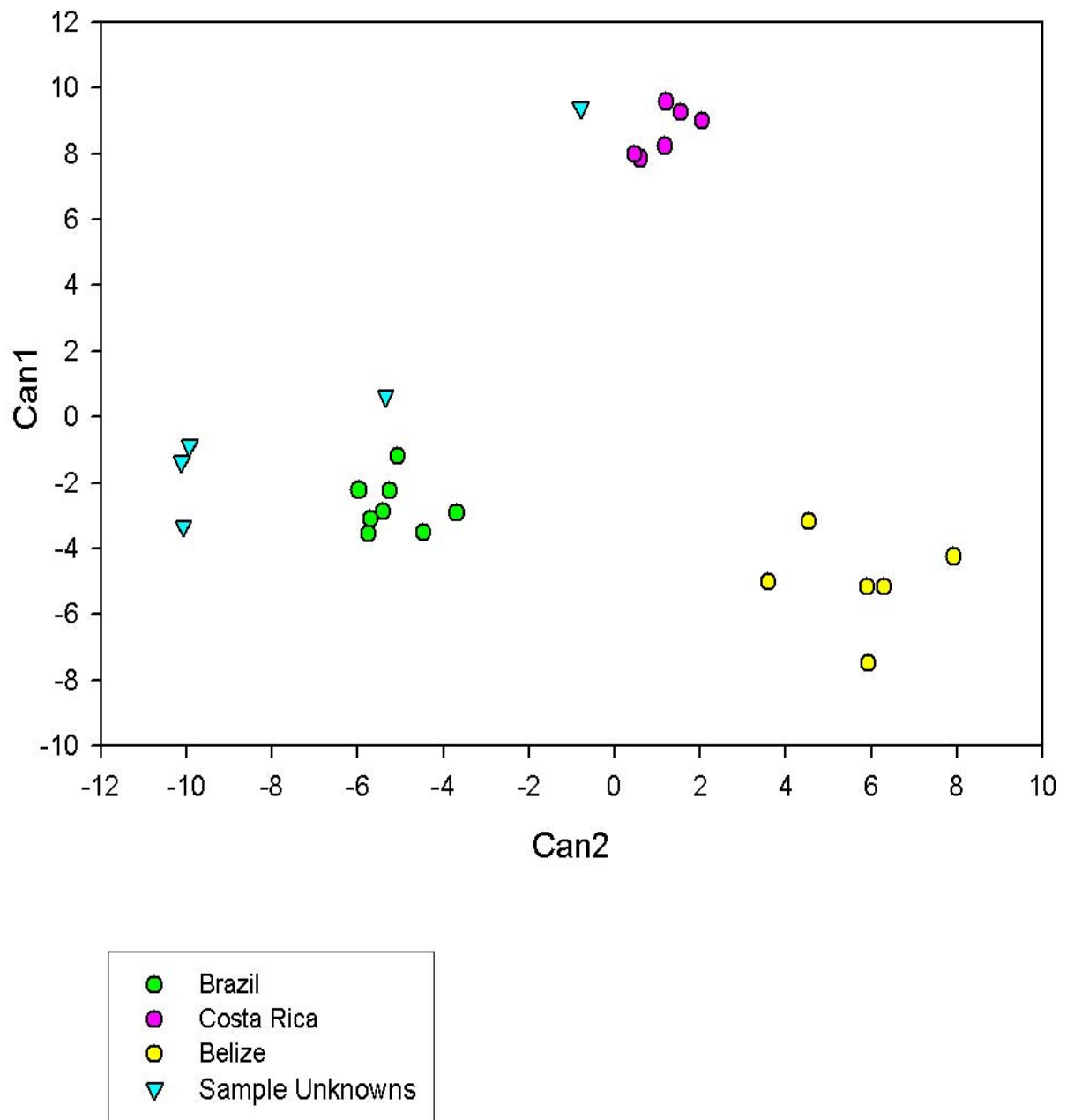


Figure 2
Canonical Plot of Apple Juice Reference Samples

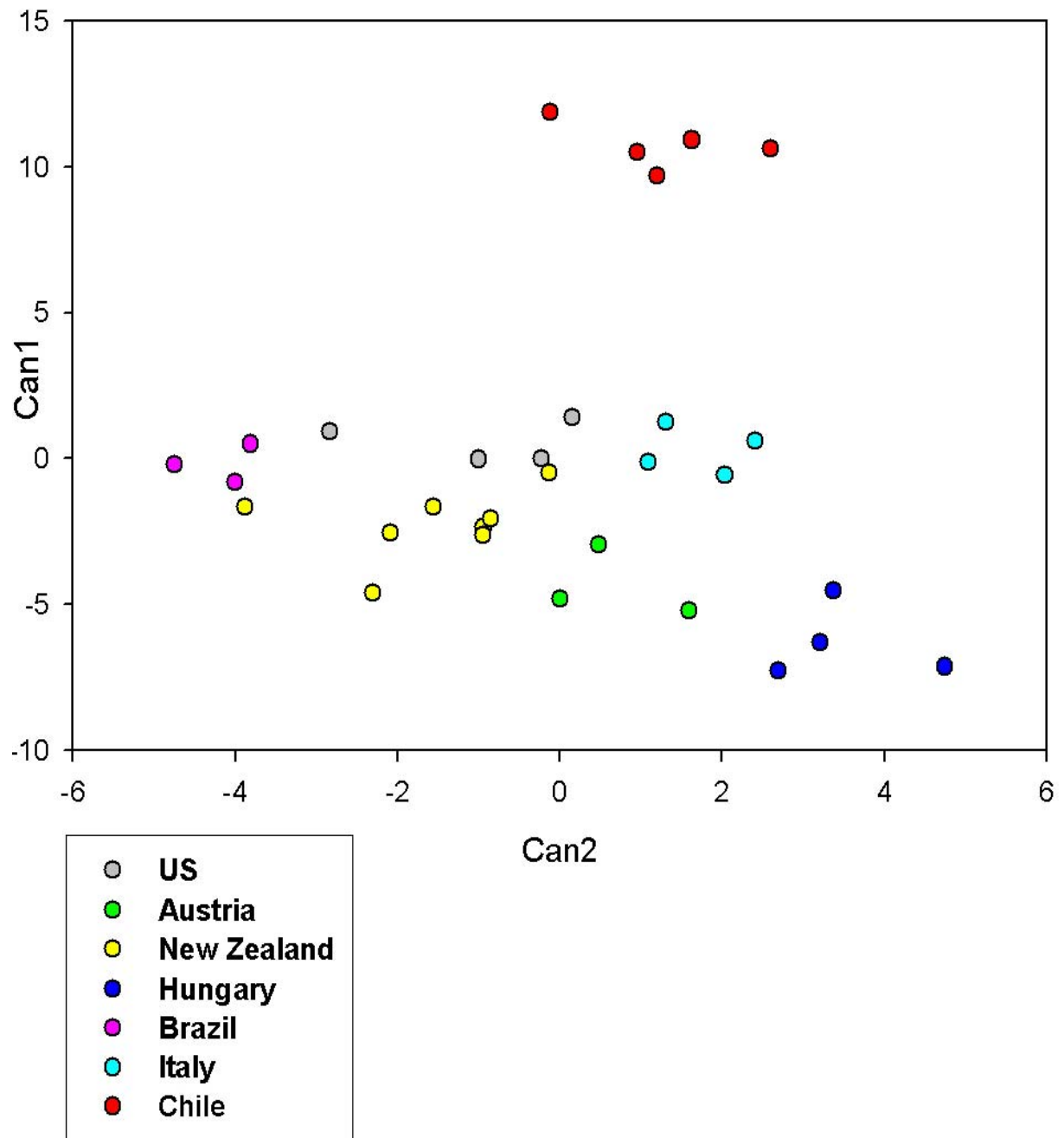
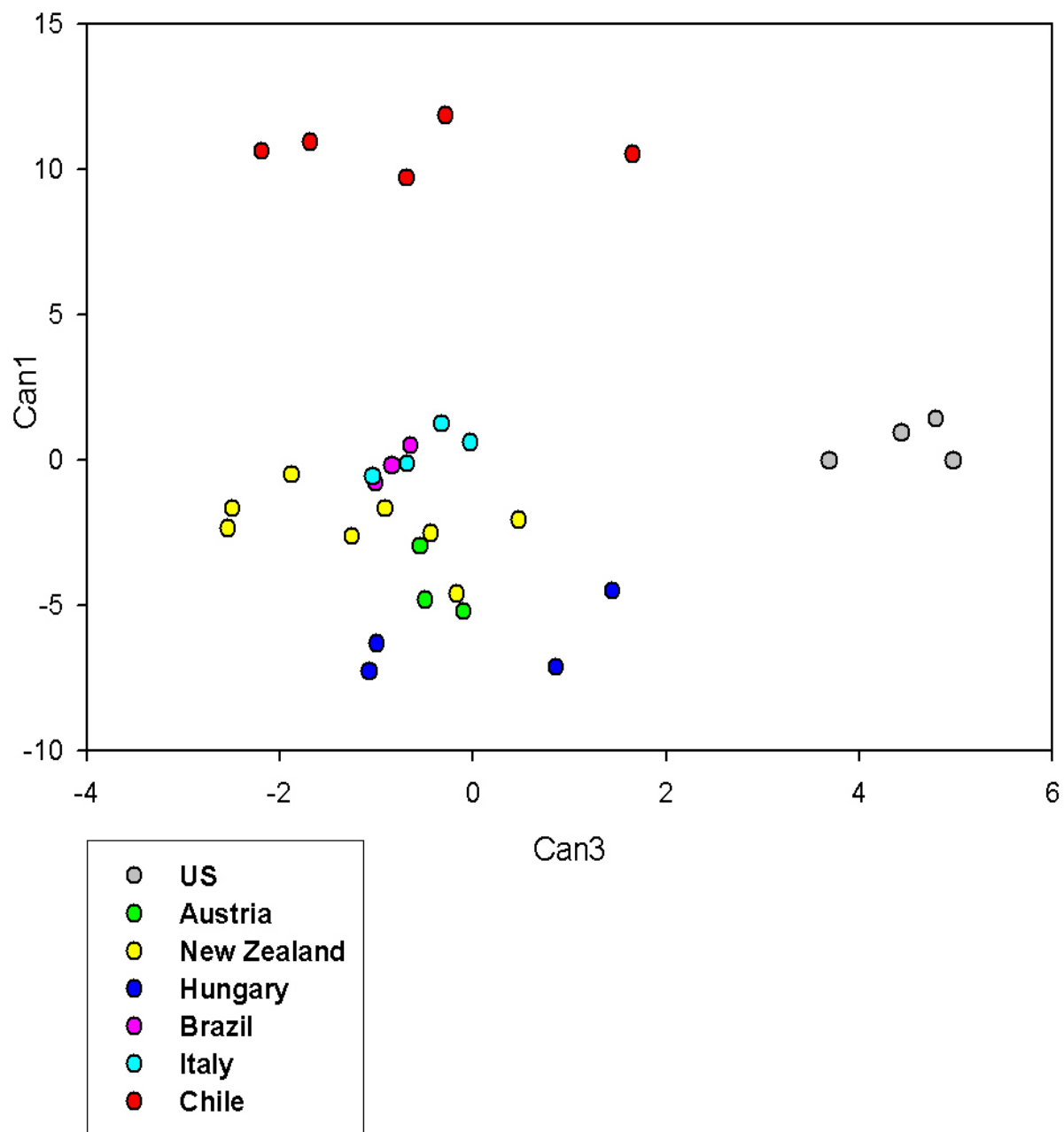


Figure 3
Canonical Plot of Apple Juice Reference Samples



THE USE OF SITE SPECIFIC NATURAL ISOTOPE FRACTIONATION NMR SPECTROSCOPY (SNIF-NMR) TO DETECT SUGAR ADULTERATION IN -FRUIT JUICES AND SPIRITS

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INTRODUCTION

Customs and Border Protection (CBP) has interests in developing the ability to determine the authenticity of imported food and food products. Examples of possible adulterations that will compromise the authenticity of imported food would be the addition of sweeteners such as cane sugar, high fructose corn syrup, or beet sugar to fruit juice, or the use of cane syrup or corn syrup in the place of agave juice in the production of tequilas and mezcals.

In the last decade a standard method of detecting such added sugar frauds was developed by the Martin research group at Universite de Nantes. This method, originally developed to detect the use of added sugar in the production of wine, employs Site Specific Natural Isotope Fractionation Nuclear Magnetic Resonance Spectroscopy (SNIF-NMR) to determine $^2\text{H}/^1\text{H}$ ratios (AOAC Official Method 995.17)^{1,2}. In general it is considered by those working in the field of food authenticity that determinations of $^{13}\text{C}/^{12}\text{C}$ ratios measured by Isotope Ratio Mass Spectrometry (IRMS) should be used along with the SNIF-NMR method in order to effectively address questions of sugar adulteration^{3,4,5}. This paper describes work done at the CBP Laboratory in Savannah, Georgia on the use of SNIF-NMR to detect added sugar in orange juice and tequila model systems. It is hoped that this study will help define the range of problems in which SNIF-NMR alone, in the absence of an IRMS capability, can be used to answer questions about added sugar in imported foods.

Due to the kinetic isotope effect and to differences in their physical properties, the stable isotopes ^{12}C and ^{13}C will be found in slightly different amounts in samples of the same chemical compound, when the samples of the compound come from plants differing in their enzymology and physiology. There are three groups of plants which can be defined based on the metabolism of their photosynthetic dark reactions; the C3 plants, the C4 plants, and the CAM plants. The dark reactions are the point at which CO_2 is first incorporated into organic compounds, and organic material from these three plant groups can be distinguished to a certain extent based on subtle differences in the $^{13}\text{C}/^{12}\text{C}$ ratios, as shown in Table 1.

Further distinctions can be made between the C3 and C4 plant groups, and within the members of the C3 plant group, when site specific $^2\text{H}/^1\text{H}$ fractionation is considered. Thus, using site specific $^2\text{H}/^1\text{H}$ ratios determined by NMR, not only can the glucose isolated from the C4 plant corn be distinguished from glucose isolated from C3 plants such as grapes or potato, but, as shown in Table 2, glucose isolated from grapes can also be distinguished from that of potatoes. In principle therefore it should be possible to determine the botanical origin of the sugar in fruit juice by direct examination of the isolated sugar by NMR. In practice however it is in fact more practical to quantitatively convert the fruit juice sugar into ethanol by yeast

supported fermentation, followed by quantitative distillation and collection of the ethanol. If care is taken to insure that these steps are performed quantitatively, so as not to fractionate the isotopes during these conversions, then the $^2\text{H}/^1\text{H}$ ratio of the ethanol methyl position will give a parameter relatable to the origin of the sugar (Table 2). The Martin research group has produced extensive literature relating the methyl $^2\text{H}/^1\text{H}$ ratio of ethanol produced by fermentation to the botanical origin of the precursor sugar, and to a large extent their work forms the basis of the approach taken in this publication^{2,3,4,7,8}.

EXPERIMENTAL

Fresh Florida orange juice (pulp free Tropicana Fresh Premium), Maker's Mark Bourbon, Knob Creek Bourbon, Bacardi Rum, Heuradura Tequila and Monta Alban Mezcal were purchased at local markets in Savannah, Georgia. Dixie Crystals, Big Chief Sugar and Crosby & Baker of Atlanta, Georgia were used as sources for authentic samples of cane sugar, sugar beet sugar and corn sugar, respectively. Authentic samples of tequilas derived from fermentation and distillation of agave juice and sugar-agave juice mixtures, and authentic samples of Magueyes Tequila and Gran Centenario Tequila, were provided by the Tequila Regulator Board of Mexico and the Mexican Customs Laboratory.

Yeast (*Saccharomyces Cerevisiae* strain K1-V1116) was purchased from Lalvin of Montreal, Canada.

Tetramethylurea (TMU) standard, with a known $^2\text{H}/^1\text{H}$ ratio measured to a precision of 0.01 ppm, was purchased from the European Commission Joint Research Center Institute for Reference Materials and Measurements, Retieseweg, Belgium (IRMM).

AOAC Official Method 995.17 (Beet Sugar in Fruit Juices) was followed in detail with minor modifications¹. In cases where ethanol samples isolated from rums, bourbons, tequilas or mezcals were to be analyzed, the fermentation step described in the method was, of course, omitted. In cases where ethanol from orange juice, sugar solutions, or mixtures of orange juice with sugar solutions were to be analyzed, 5 grams of yeast were added to each incubation to initiate fermentation. (We found that the amount of yeast specified in the AOAC Official Method, 0.3 gm, to be inadequate. We believe that the quantity of 0.3 gm found in the published AOAC method maybe a typographical error.) The incubations were set up so that the total initial sugar in each 600 gm fermentation was 9% sugar by weight, the sugar concentration found in single strength pulp free Tropicana Fresh Premium orange juice. Progress of the fermentations was followed by monitoring the disappearance of carbohydrate using the "Clinitest" assay kit purchased from Bayer.

Quantitative distillations of the ethanol produced in the fermentations were done using special computer controlled stills purchased from Eurofins Scientific of Nantes, France. Ethanol concentrations in the distillates were determined using a Anton Paar DMA 5000 Density Meter.

Deuterium NMR Spectra were taken on a Bruker AVANCE DRX 500 MHz spectrometer equipped with a 10 mm ^2H probe and ^{19}F lock circuitry. Instrumental parameters were as indicated in the AOAC method. WALTZ16 composite pulse decoupling was used to achieve ^1H decoupling.

The $^2\text{H}/^1\text{H}$ for the ethanol methyl position was calculated as:

$$(^2\text{H}/^1\text{H})_{\text{methyl}} = 1.5866 \times (A_{\text{methyl}}/A_{\text{TMU}}) \times (M_{\text{TMU}}/M_{\text{Ethanol}}) \times (1/F_{\text{ethanol}}) \times (^2\text{H}/^1\text{H})_{\text{TMU}}$$

$A_{\text{methyl}}/A_{\text{TMU}}$ = ratio of CH_2D and TMU peak areas

$M_{\text{TMU}}/M_{\text{Ethanol}}$ = ratio of TMU and ethanol masses

F_{ethanol} = fraction of ethanol in distillate as measured by Paar density meter

$(^2\text{H}/^1\text{H})_{\text{TMU}}$ = ratio for TMU standard

Accuracy of the NMR measurements was validated by determinations of ethanol methyl position $^2\text{H}/^1\text{H}$ ratios made on standard samples of corn sugar ethanol, grape sugar ethanol and sugar beet sugar ethanol purchased from the European Commission Joint Research Center Institute for Reference Materials and Measurements, Retieseweg, Belgium (IRMM).

Measurements of ethanol $^{13}\text{C}/^{12}\text{C}$ ratios were made using standard IRMS methods⁹ by Mike Lott and Craig Cook of the Stable Isotope Resource For Environmental Research at the University of Utah (SIRFER). The $^{13}\text{C}/^{12}\text{C}$ data are included here by the permission of Professor James Ehleringer, Director of SIRFER.

RESULTS AND DISCUSSION

1. The Relationship of Signal-to-Noise to Precision and Sample Analysis Time

Figure 1 shows a typical ^2H NMR spectrum for an ethanol sample. The ethanol natural abundance CH_2D , CHD , OD peaks can be seen at 1.1, 3.5 and 4.75 ppm, respectively. The peak at 2.75 ppm is the natural abundance CH_2D peak of the TMU standard. Since the $^2\text{H}/^1\text{H}$ ratio for the TMU methyl position is known, the isotope ratio of the ethanol methyl can be calculated from the ratio of the intensities, or peak heights, of the ethanol and TMU CH_2D NMR peaks, as indicated in the equation described in the preceding section. It is important to note that the methyl $^2\text{H}/^1\text{H}$ ratio differences of interest may be fairly small. The difference in the methyl position $^2\text{H}/^1\text{H}$ ratio for ethanol produced by the fermentation of pure fresh orange juice is only 4 to 6% different from that of the ethanol produced by the fermentation of a cane sugar solution, and it is therefore necessary to be concerned about the precision of the measurements.

It is easy to obtain precisions of one part per thousand or better for the weight and ethanol purity measurements used in calculating the methyl $^2\text{H}/^1\text{H}$ ratios. Thus the uncertainty of the $^2\text{H}/^1\text{H}$ ratio determinations will be limited by, and inversely related to, the signal-to-noise ratio of the NMR spectrum. Since the signal-to-noise ratio will increase with the square root of the number of FIDs averaged, there is a fundamental trade off between the precision of a $^2\text{H}/^1\text{H}$ ratio determination and the sample analysis time. For each four fold increase in the number of FIDs averaged per spectrum, there is roughly a two fold decrease in the standard deviation for the $^2\text{H}/^1\text{H}$ ratio determination calculated from a set of 10 spectra (see data in Table 3).

The AOAC method recommends that the FIDs be acquired with enough signal averaging to give a signal-to-noise ratio of 150 or better for the ethanol methyl peak transformed with an exponential apodization using $LB = 2 \text{ Hz}^{-1}$, an acquisition requiring 15 minutes on the 500 MHz instrument. The method indicates that ten spectra should be averaged for each $^2\text{H}/^1\text{H}$ ratio determination, making for a total analysis time of roughly 3 hours per sample, including setup. This procedure results in a standard deviation of about one part per hundred, which we considered unacceptable for our purposes. We obtained a good trade off between analysis time and precision by acquiring FIDs for one hour each, thus achieving a signal-to-noise ratio of 300 or better, and averaging values calculated from six spectra for each sample. This corresponds to a total acquisition time on the NMR of six hours per sample, but results in a standard deviation of one part in two hundred.

2. Bourbon and Rum: An Illustration

Methyl position $^2\text{H}/^1\text{H}$ ratios for ethanol isolated by distillation from bourbon and rum are shown in Table 4, along with ratios for ethanol produced in the laboratory by the fermentation of Dixie Crystals (100 % cane sugar sucrose) and Crosby & Baker Corn Sugar (100 % corn sugar dextrose). The ratio for fermented barley shown in Table 4 was found in the literature⁸. Initially we found it interesting that while the rum $^2\text{H}/^1\text{H}$ value closely matched that of the fermented cane sugar ethanol, which seems natural as rum is made by the fermentation of cane sugar, the bourbons showed ratios considerably lower than that of our fermented corn sugar. However, although bourbon is commonly thought of as “corn whiskey”, in fact by regulation bourbon is produced from a mixture of corn with other grains, usually the C3 grains barley, rye or wheat. To be a bourbon a whiskey must have at least 51% of its ethanol derived from the fermentation of corn¹⁰. Without $^{13}\text{C}/^{12}\text{C}$ data determined by IRMS, it is difficult to quantitatively determine the amount of C3 grain used in the production of the Marker’s Mark and Knob Creek bourbons. However it is clear from the NMR data that the Marker’s Mark bourbon was made using more C3 grain than the Knob Creek bourbon.

3. Detection of Added Sugar Used to Sweeten Orange Juice

Table 5 presents SNIF-NMR methyl $^2\text{H}/^1\text{H}$ ratios for ethanols produced by the fermentation of fresh Florida orange juice and orange juice adulterated with beet sugar or cane sugar. For purposes of comparison IRMS $^{13}\text{C}/^{12}\text{C}$ ratios taken for selected samples are also presented in Table 5 and Figure 2. Based on this data the authors feel it is fair to say that SNIF-NMR alone, in the absence of an IRMS capability, can detect significant amounts of cane or beet sugar adulteration in fruit juice. It is also worth noting that only determinations of $^2\text{H}/^1\text{H}$ can detect the presence of beet sugar, as the $^{13}\text{C}/^{12}\text{C}$ ratios for C3 fruit sugar and C3 sugar beet sugar are pretty much the same. Further it would appear from the data in Table 5 that the ^2H NMR measurements can be used to semi-quantitatively estimate the amount of adulteration. The $^2\text{H}/^1\text{H}$ ratio for a 50% adulteration of orange juice with cane sugar is roughly half way between the values for pure orange juice and pure cane sugar.

However it is very important to remember that the data in Table 5 and Figure 2 are for model systems mixed in the laboratory, rather than real samples of imported fruit

juice. This is a significant difference. The methyl $^2\text{H}/^1\text{H}$ ratios determined for fresh unadulterated fruit juice will be strongly dependant on the geographic origin of the fruit. Thus, in a study conducted by the Florida Citrus Commission and EUROFINS Scientific, the mean ethanol methyl $^2\text{H}/^1\text{H}$ ratios for fresh orange juice from Brazil, Israel and Florida were found to be 104.2, 107.0 and 104.8 ppm, respectively¹¹. While the mean methyl $^2\text{H}/^1\text{H}$ ratios for grape juice from France, California and South Africa are 100, 104 and 108 ppm, respectively¹². This geographic variation may prove useful in country of origin studies, however it presents a major limitation on the use of SNIF-NMR in the context of the detection of added sugar adulteration. In order to correct for geographic variation in SNIF-NMR studies EUROFINS Scientific provides a software package utilizing data from twenty orange juice producing countries and nine apple juice producing regions. However the use of this software will give only an indication of whether or not an imported fruit juice is suspect of added sugar adulteration, the NMR determined $^2\text{H}/^1\text{H}$ ratios are not used as a quantitative measure of the degree of adulteration.

4. Detection of Added Sugar Used in the Production of Tequilas and Mezcal

Authentic “Tequila” must be made from the juice of the blue agave plant grown in a specially designated region in the Mexican states of Jalisco, Michoacan, Guanajuato, Nayarit and Tamaolipas. Drink produced by the fermentation and distillation of the white agave elsewhere in Mexico is labeled as “Mezcal”¹³. The musts used in the production of authentic “Tequila” may legally be enriched by up to 49% of total sugar with non-agave sugars, such as cane syrup or high fructose corn syrup. However to be labeled as “Tequila 100% Agave” the product must be made with out the addition of non-agave sugar¹³.

Recently the agave crop has been attacked by pathogens and insects. The 2001 harvest produced only 270,000 tons of agave flesh, a very poor yield compared to the 730,000 tons produced during the 2000 harvest¹⁴. At the same time, driven by an expanding demand from Western Europe, tequila production has in fact increased from 7.5 to 21 million cases over the last ten years¹⁴. This suggests that cane or corn syrups may be being used as substitutes for agave juice.

This problem was brought to our attention by the Canada Customs and Revenue Agency (CCRA) and the Tequila Regular Board of Mexico (TRB). The TRB provided to our laboratory, via the CCRA, authentic samples of tequila made from blue agave, as well samples produced from agave juice partly adulterated with cane sucrose or corn fructose, and samples of two commercial tequilas. SNIF-NMR data for the ethanol quantitatively isolated from these samples are presented in Table 6.

Disappointingly no distinction could be made, using site specific $^2\text{H}/^1\text{H}$ NMR data alone, between 100 % agave tequila, and tequila produced using cane or corn sugars. Although the ethanol methyl $^2\text{H}/^1\text{H}$ ratio for sample 3 in Table 6 is slightly higher than that for the other tequila samples, this does not appear to be a significant result. We have included these results for the tequila added sugar problem here in order to illustrate the limitations of the SNIF-NMR technique when it is used as the sole test technology for these type of studies.

CONCLUSIONS

In the detection of added sugar, SNIF-NMR measurements of ethanol methyl $^2\text{H}/^1\text{H}$ ratios and IRMS measurements of $^{13}\text{C}/^{12}\text{C}$ ratios are complimentary techniques. Each technique has its own strengths. Site specific $^2\text{H}/^1\text{H}$ ratios can be used to detect the addition of sugar from a C3 source to a food product from a C3 source. An important example of this being the adulteration of orange juice or apple juice with sugar beet sugar. However while the use of SNIF-NMR as a quantitative tool is limited by the variations of the ethanol methyl $^2\text{H}/^1\text{H}$ ratio arising from the geographic origin of the fruit juice and the sugar, IRMS measurements of ethanol $^{13}\text{C}/^{12}\text{C}$ ratios have been found to be largely independent of geographic origin^{4,11,15}. Thus measurement of $^{13}\text{C}/^{12}\text{C}$ ratios is a more precise way to determine the botanical origin of sugars, without the complications of geographic variations, when the question being asked relates to a distinction between C3 plant and C4 plant sugar sources. An important example of this being the adulteration of fruit juice with cane sugar or high fructose corn syrup.

The added sugar tequila problem is another illustration of the relative strengths of the two techniques. The ethanol methyl $^2\text{H}/^1\text{H}$ ratio for sample 3, ethanol distilled from "tequila" made by fermentation of 51% blue agave sugar and 49% cane sugar, was slightly higher than the value for either authentic blue agave sugar ethanol or our authentic cane sugar ethanol. However it is uncertain what this might mean. This result might be indicative of a geographic variation between the cane sugar used in the preparation of the tequila in Mexico, and our authentic "Dixie Crystal" cane sugar. It would be useful to have $^{13}\text{C}/^{12}\text{C}$ ratio data for sample 3 to clarify this point. At the bottom line the authors were unable to distinguish, using SNIF-NMR alone, whether ethanol in the various tequila samples had originated from the fermentation of agave sugar or from mixtures of agave sugar with corn sugar or cane sugar. And it is important to note that workers at Thermo FINNIGAN have been able to solve the added sugar tequila problem using a combination of ethanol $^{13}\text{C}/^{12}\text{C}$ ratios and ethanol $^{18}\text{O}/^{16}\text{O}$ ratios, both measured using isotope ratio mass spectrometry (IRMS)¹⁶.

It is clear that no single technique can address all the specific questions being asked in the area of added sugar adulteration. In Table 7, a correlation between the particular type of added sugar question and the analytical technique of choice is offered based upon this article.

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15. Martin, P.D., Zhang, B.-L. and Martin, G.J. (1994) *American Journal of Enology and Viticulture* 45 79-85
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Table 1. Relation of ^{13}C Incorporation to the Metabolism of the Photosynthetic Dark Reactions*

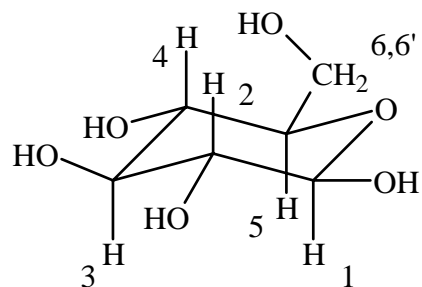
<u>Dark Reaction Metabolism</u>	<u>Food Plants</u>	<u>$\delta_{\text{pdb}}^{13}\text{C}/^{12}\text{C}^{**}$</u>
C3 (Calvin Cycle)	oranges, apples, grapes sugar beets, wheat, rice oats, barley, malt, rye potato, soybean	-32 to -24
C4 (Hatch-Slack Pathway)	corn, sugar cane, millet	-16 to -10
CAM (Crassulacean Acid Metabolism)	pineapple, agave	-30 to -12

from reference 6*

$$^{**}\delta_{\text{pdb}} = (R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}} \times 1000 \quad R = ^{13}\text{C}/^{12}\text{C}$$

Standard is Pee Dee Belemite

Table 2. Relation of Site Specific $^2\text{H}/^1\text{H}$ Ratios (PPM)
to Botanical Origin in Glucose and Ethanol *



<u>Plant</u>	<u>Glucose</u>			<u>Ethanol Methyl</u>
	<u>1</u>	<u>2,3,4,5</u>	<u>6,6'</u>	
Corn	143	154	154	110
Grape	114	160	145	102
Potato	109	150	131	96
from reference 7 *				

Table 3. Precision of SNIF-NMR Measurements of Ethanol Methyl $^2\text{H}/^1\text{H}$ Ratio. Dependence of Standard Deviation (S.D.) on the Number of FID Scans Averaged per Spectrum (N.S.).			
N.S.	<u>128</u>	<u>512</u>	<u>2048</u>
Experiment Time One Spectrum (hours)	0.25	1	4
Experiment Time Ten Spectra (hours)	2.5	10	40
S.D.* (ppm) IRMM Corn Ethanol Standard	1.2	0.4	0.2
S.D.* (ppm) IRMM Grape Ethanol Standard	0.8	0.4	0.2
S.D.* (ppm) IRMM Sugar Beet Ethanol Standard	0.9	0.4	0.1
* Standard Deviation Calculated for Ten Spectra			

Table 4. SNIF-NMR Study of Ethanol Isolated from Fermented Sugars, Rum and Bourbon by Quantitative Distillation

<u>Ethanol Source</u>	<u>Ethanol Methyl $^2\text{H}/^1\text{H}$ (ppm)</u>	<u>S.D.</u>
Fermented Cane Sugar ("Dixie Crystals")	110.6	0.5
Fermented Corn Sugar (Dextrose from Crosby & Baker)	110.2	0.9
Bacardi Rum	111.0	0.7
Maker's Mark Bourbon	106.4	0.7
Knob Creek Bourbon	108.0	0.6
Fermented Barley*	103.6	
* from reference 8		

Table 5. SNIF-NMR Detection of Added Sugar Adulteration of Fresh Orange Juice

<u>Sugar Source in Fermentation</u>	<u>Ferm.#</u>	<u>NMR Sample</u>	<u>Ethanol Methyl $^2\text{H}/^1\text{H}$ (ppm)</u>	<u>S.D.</u>	<u>$\delta^{13}\text{C}/^{12}\text{C}$</u>
Cane Sugar	1	A	110.8	0.5	
"	1	B	110.4	0.4	
"	2	A	110.4	0.5	-12.17
"	2	B	111.0	0.4	"
0.5 Cane Sugar 0.5 Orange Sugar	1	A	108.6	0.6	
"	1	B	108.4	0.4	
"	2	A	107.0	0.5	-18.11
"	2	B	108.6	0.5	"
Orange Sugar	1	A	106.4	0.5	
"	1	B	106.5	0.7	
"	2	A	106.5	0.6	-26.65
"	2	B	106.8	0.4	"
0.5 Beet Sugar 0.5 Orange Sugar	1	A	96.5	0.5	
"	1	B	98.2	0.5	
"	2	A	97.2	0.5	-26.27
"	2	B	97.4	0.5	"
Beet Sugar	1	A	89.9	0.4	
"	1	B	89.8	0.4	
"	2	A	92.0	0.6	-26.74
"	2	B	90.3	0.7	

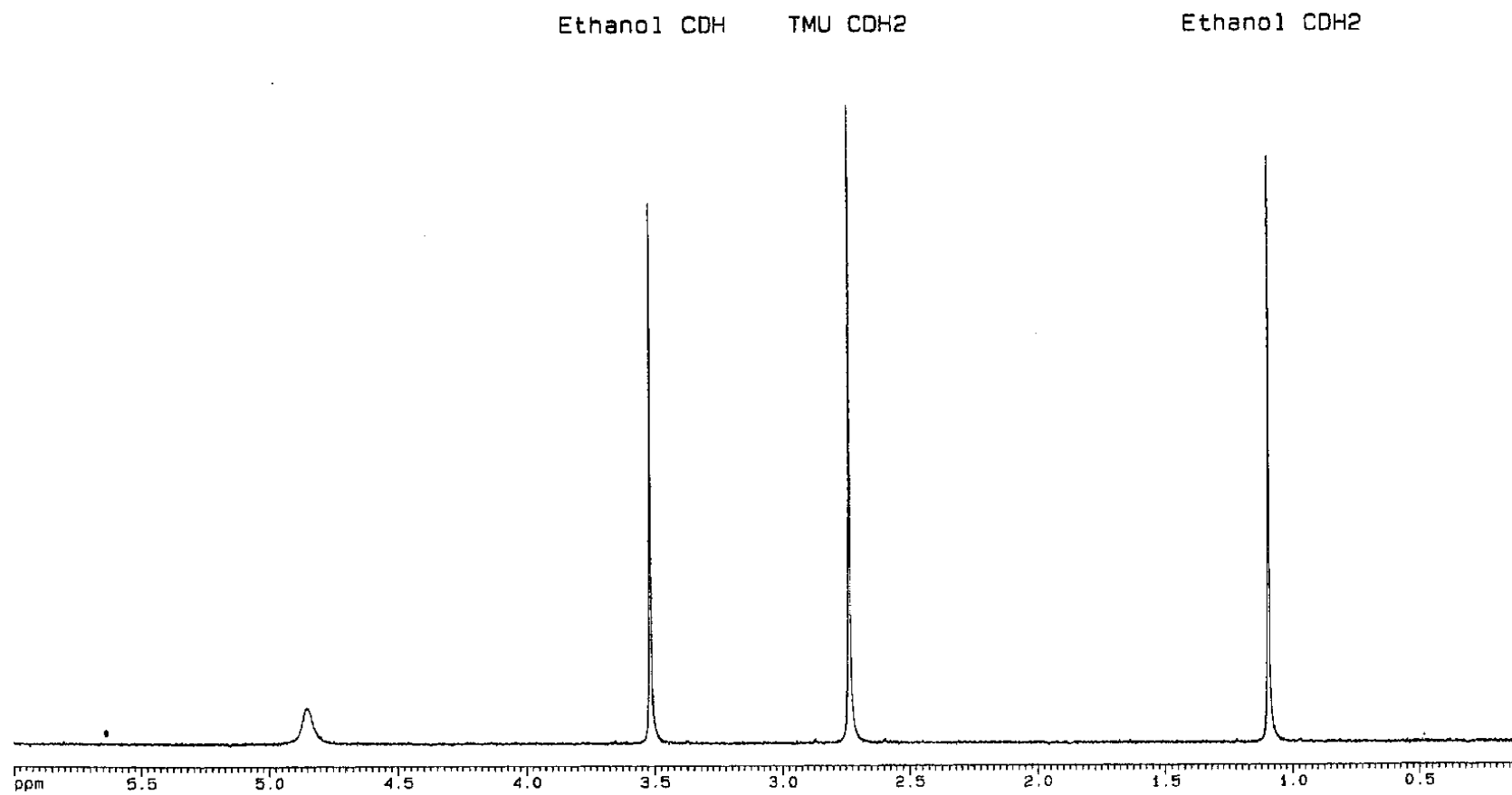
Table 6. Ethanol Methyl $^2\text{H}/^1\text{H}$ Ratios for Commercial Tequilas and Model Samples of Adulterated Tequila

<u>Sample</u>	<u>NMR Sample</u>	<u>Methyl $^2\text{H}/^1\text{H}$ (ppm)</u>	<u>Standard Deviation</u>
Model Sample #1*	A	110.0	0.3
1.0 Agave	B	110.8	0.8
Model Sample #2*	A	110.6	0.7
0.55 Agave	B	110.0	0.3
0.45 Fructose Corn Syrup			
Model Sample #3*	A	112.2	0.6
0.514 Agave	B	112.1	0.5
0.486 Cane Syrup			
Magueyes Tequila*	A	111.5	0.5
"	B	111.4	0.3
Gran Centenario Tequila*	A	109.8	0.4
"	B	109.3	0.6
Heuradura Tequila **	A	111.1	0.5
"	B	111.9	0.9
Monta Alban Mezcal **	A	109.3	0.6
"	B	109.4	0.4
From Tequila Regulator Board of Mexico *			
Purchased in Savannah, Georgia **			

Table 7. Method of Choice for Detection of Various
Forms of Added Sugar Adulteration

<u>Suspected Adulteration Other</u>	<u>SNIF-NMR</u>	<u>IRMS ¹³C/¹²C</u>	<u>IRMS ¹⁸O/¹⁶O</u>	
Beet Sugar Adulteration of Orange Juice	X			
Beet Sugar Adulteration of Apple Juice	X			
Percent C3 Plant Ethanol in Bourbon Whiskeys	X	X		
Cane or Corn Sugar Adulteration of Fruit Juice	X	X		
Cane or Corn Sugar Used in the Production of Tequila		X	X	
Pear Juice Adulteration of Apple Juice				X

Figure 1: Deuterium NMR Spectrum of Distillate Ethanol Sample
(65% Distillate Ethanol, 32% TMU, 3% C6F6 by weight)



**Figure 2 Models for Added Sugar
Adulteration of Fresh Florida Orange Juice
(Data from Table 5)**

